if any, reorientation of the enzyme-bound steroid with respect to the direction of attack need be postulated. Further, no separation of the individual enzymatic activities of the previously proposed multienzme pathway has ever been reported. Final proof awaits the isolation and characterization of those components of placental microsomes responsible for aromatization.

Until now, the term "aromatase" has been rather loosely applied to the preparation of placental microsomes capable of converting androstenedione to estrone. The evidence presented here justifies the application of this term to the enzyme which catalyzes the aromatization of a variety of steroids.

Supplementary Material Available

Plots of the kinetic and binding data referred to in the text (10 pages). Ordering information is given on any current masthead page.

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Density Differences between Membrane and Secreted Immunoglobulins of Murine Splenocytes†

Ulrich Melcher*,‡ and Jonathan W. Uhr

ABSTRACT: The buoyant densities of mouse immunoglobulins were determined by isopycnic centrifugation in phosphate-buffered cesium chloride using β-galactosidase as marker. The buoyant densities of IgG, TEPC 15 IgA, secreted IgM, and MOPC 104E IgM were consistent with their carbohydrate contents both in the presence and the absence of the nonionic detergent, Nonidet P-40. Intracellular IgM from spleen cell lysates had a buoyant density corresponding to a carbohydrate content of 6%. Membrane IgM from detergent lysates of spleen cells was less dense than either intracellular or secreted IgM in the presence of detergent. The IgD-like membrane molecules were more dense than membrane IgM.

The physical and chemical properties of membrane immunoglobulins are of great interest because of their central role in the humoral immune response (Siskind and Benacerraf, 1969). The membrane Ig's[1] are primarily of the IgM and IgD classes (Melcher et al., 1974; Fu et al., 1974; Abney and Parkhouse, 1974. The identification of the second class of murine membrane Ig as IgD is based on the absence of antigenicity of the H chain with anti-μ, -γ, or -α, its prominence on lymphocyte surfaces in contrast to its paucity in serum, an apparent molecular weight of its H chain between μ and γ, and a carbohydrate content similar to IgM. All of these properties are shared with human IgD (Spiegelberg, 1972). Membrane Ig's are probably integral membrane proteins (Singer and Nicolson, 1972), both since detergents are needed to extract them (Kennel and Lerner, 1973; Vitetta et al., 1971) and since membrane Ig's require the continued presence of detergent for solubility (Melcher et al., 1975).

Membrane IgM differs from secreted IgM in several respects. The membrane IgM is monomeric rather than pentameric. The μ chain of mouse membrane IgM moves slightly slower than that of the secreted IgM on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Melcher and Uhr, 1976; Lisowska-Bernstein and Vassalli, 1975). Monomeric secreted IgM derived from 19S IgM by partial reduction has a considerably greater mobility on sodium dodecyl sulfate–

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[1] Abbreviations used are: Ig, immunoglobulin; H, heavy chain; L, light chain; PBS, phosphate-buffered saline; NP40, Nonidet P-40.

polyacrylamide gel electrophoresis than does the monomeric membrane IgM (Melcher and Uhr, 1976). It is not clear which, if any, of these observations are directly related to the membrane binding of Ig.

Membrane IgM of lymphocytes should also be compared with intracellular IgM of plasma cells since membrane IgM may be derived from a similar molecule in the lymphocyte (Knopf, 1973) rather than from secreted IgM. Both membrane and intracellular IgM exist in the monomeric form (Vitetta et al., 1971; Melcher and Uhr, 1976; Parkhouse and Askonas, 1969). Intracellular IgM in the mouse lacks the terminal sugars, particularly fucose, galactose, and sialic acid, which are present in secreted IgM (Melchers, 1973). No clear data on the carbohydrate content of membrane IgM are available, but evidence from sodium dodecyl sulfate–polyacrylamide gel electrophoresis suggests that membrane IgM is fully glycosylated (Melcher and Uhr, 1976). Comparisons of human membrane IgD with serum IgD have not yet been performed. Such comparisons are not yet possible in the mouse since a soluble IgD-like molecule has not yet been identified.

In order to clarify some of the above issues, we sought to further characterize membrane Ig by isopycnic centrifugation in cesium chloride. This technique is sensitive to slight differences in density of the sedimenting particle. The density of an immunoglobulin particle could be raised by the presence of extra carbohydrate, or it could be lowered by the strong binding of detergent to it. Strong binding of detergents is a property of integral membrane proteins (Helenius and Simons, 1975). Isopycnic centrifugation of proteins has been used to study their physical properties, and, using heavy isotopic precursors, their biosynthesis (Filner and Varner, 1967). Isopycnic centrifugation has already been used to separate glycoproteins of differing carbohydrate content (Creath and Denborough, 1970 Robinson and Monsey, 1971). In this article we will first establish the validity of isopycnic centrifugation in studying Igs of different carbohydrate content. We then present data which suggest that membrane IgM may bind detergent.

Methods

The procedures for radioiodination and lysis of spleen cell suspensions from 6–12 week old Balb/C mice have been previously described (Vitetta et al., 1971; Melcher and Uhr, 1973). Incubation of cells with [^{3}H]tyrosine and the preparation of cell supernatants and lysates from such cells were also as previously described (Melcher and Uhr, 1973; Vitetta and Uhr, 1972). ^{3}H-Labeled cell supernatants were partially reduced with dithioerythritol and alkylated with iodoacetamide according to the procedure of Askonas and Parkhouse (Melcher and Uhr, 1976; Askonas and Parkhouse, 1971).

Mouse γ-globulin (Miles Pentex, Kankakee, Ill.), TEPC 15 IgA (Bionetics, Kensington, Md.), and MOPC 104E IgM (prepared from ascites fluid by Drs. M. Boesman and D. Hart) were iodinated in solution with lactoperoxidase. The reaction mixture contained in 0.25 ml of PBS: 0.10 mg of protein, 20 μg of lactoperoxidase, 200 μCi of Na^{125}I, and 0.22 μmol of H_2O_2. After 30 min at room temperature, the reactions were chilled and extensively dialyzed against PBS. Radiopurity of the product was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, which was performed as previously described (Vitetta et al., 1971; Melcher and Uhr, 1976).

CsCl Centrifugation of Immunoglobulins. Polyallomer centrifuge tubes were filled with 0.87 ml of saturated CsCl (20 °C), sample and PBS were added to 1.13 ml, and 1.0 ml of water. The samples used were lysates of radiolabeled cells, ^{125}I-labeled immunoglobulins, or ^{3}H-labeled cell supernatants. β-Galactosidase (50 μg) was included in most samples as reference. A layer of paraffin oil was laid over the aqueous sample. The tubes were then centrifuged 65 h at 35 000 rpm in an SW 56 rotor (Beckman Instruments, Palo Alto, Calif.) at 5 °C. The tubes were then punctured at the bottom and two- or three-drop fractions collected from the middle of the gradient. Densities were determined from refractive indexes of selected fractions. β-Galactosidase was assayed on aliquots of the PBS diluted fractions by measuring the hydrolysis of *O*-nitrophenyl β-D-galactopyranoside (Pardee et al., 1959). For analysis of ^{125}I-labeled membrane Ig and ^{3}H-labeled secreted Ig, fractions were diluted with 0.2 ml of PBS and reacted with either rabbit anti-κ, rabbit anti-μ, or rabbit anti-$\phi\chi$174 sera (Melcher et al., 1974). Complexes were precipitated with goat anti-rabbit Ig as previously described (Vitetta et al., 1971; Melcher and Uhr, 1976). For analysis of ^{125}I-labeled mouse Ig proteins, fractions were counted directly. In some cases the results of several gradients were plotted together on the same graph by superimposing the β-galactosiase profiles. When this was done, the density points obtained from the refractive index fell on one line.

Results

Isopycnic Centrifugation of Serum Ig's. The buoyant density of a hydrodynamic particle is a function of the buoyant density of its component parts (Ifft and Vinograd, 1966). For Ig, these parts include the polypeptide chains, the oligosaccharide chains, and water and ions bound to these components. In order to make useful comparisons among immunoglobulins it is necessary that the buoyant density of the polypeptide (with its associated water and ions) not be significantly different from one immunoglobulin to another. We therefore compared the buoyant densities of four mouse immunoglobulin preparations of differing carbohydrate content. Normal mouse γ-globulin was used as a source of IgG. TEPC 315 IgA, obtained commercially, and MOPC 104E IgM are monoclonal mouse myeloma proteins. In addition, supernatants of mouse spleen cells incubated for several hours with [^{3}H]tyrosine were used as a source of IgM. We trace labeled the mouse γ-globulin and the myeloma proteins with ^{125}I (less than 0.1 g-atom of iodine per mol of Ig).

Each of these Ig's was spun to equilibrium in a separate tube containing cesium chloride, PBS, and β-galactosidase. The β-galactosidase profiles of each gradient were superimposed and the density profiles of the Ig's were plotted together (Figure 1). The differences in density between the peak positions of β-galactosidase and the Ig preparations are presented in Table I, as are the apparent densities of the peaks. Because of variation from experiment to experiment in the reading of refractive indices, an uncertainty of 0.005 g/cm^3 is associated with the density values. This uncertainty is reduced when two peaks in the same gradient are compared. Tailing of radioactivity on the less dense side of the peaks of IgG, TEPC 15 IgA, and [^{3}H]IgM occurred. For the [^{3}H]IgM the tailing may be due to a 10–20% contamination by IgG as determined by immunoprecipitation. The tailing of the IgG and IgA peaks was not investigated. The Ig preparations are seen to increase in density in the order: IgG, TEPC 15 IgA, [^{3}H]IgM, and MOPC 104E IgM.

Since isopycnic centrifugation of membrane Ig's can only be performed in the presence of detergent, it was necessary to ascertain that the above relative density positions were not altered by the presence of detergent. Accordingly, the same four Ig preparations were centrifuged similarly but with 0.5%

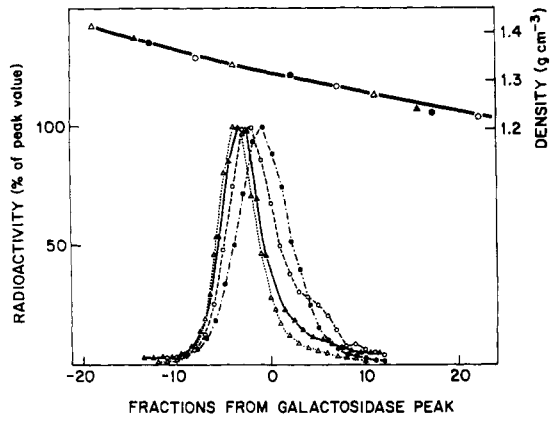

FIGURE 1: Density gradient centrifugation of Ig's in the absence of detergent. ^{125}I-Labeled mouse γ-globulin (solid circles, dashed dotted line), ^{125}I-labeled myeloma IgA (open circles, dashed line), ^{3}H-labeled secretion of mouse spleen cells (solid triangles, solid line), and ^{125}I-labeled myeloma IgM (open triangles, dotted line) were centrifuged in separate gradients with β-galactosidase. Three drop fractions were collected. ^{125}I profiles were counted directly. The ^{3}H profile represents the difference between precipitation with rabbit anti-κ and rabbit anti-$\phi\chi$ (control). All fractions were normalized to the peak fraction (100%). The peak of enzyme activity was in each case assigned to fraction number 0.

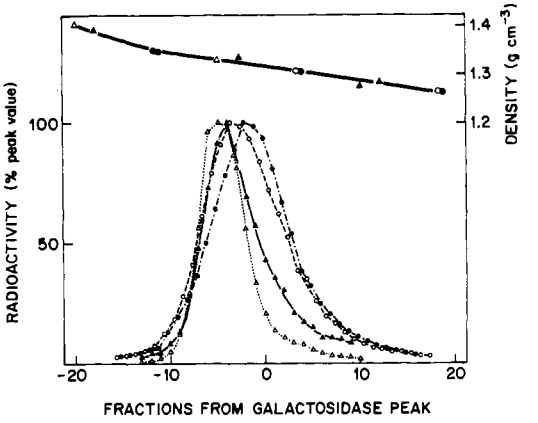

FIGURE 2: Density gradient centrifugation of Ig's in the presence of NP40. Details are as described in Figure 1.

NP40 added to the samples. When the enzyme activity profiles were superimposed, the density distributions illustrated in Figure 2 were obtained. The profiles appear broader than those in Figure 1 since detergent decreased the volume of each fraction. The positions of the four Ig preparations relative to one another were unchanged. The trailing of radioactivity on the less dense side of the peaks is also apparent for TEPC 15 IgA and [^{3}H]IgM.

The differences in density between the peak positions of β-galactosidase and Ig preparations are presented in Table I, part A. Since the density of β-galactosidase is not affected by detergent (Meunier et al., 1972) it is used as a reference point. The densities of TEPC 15 IgA, [^{3}H]IgM, and MOPC 104E IgM were not significantly lowered by detergent. IgG was slightly more dense in the presence of NP40 than in its absence. The differences in Ig density are as expected from knowledge of their carbohydrate contents. MOPC 104E IgM is 11.7% carbohydrate as calculated from the data of Robinson et al. (1974). The majority of mouse IgM's differ from MOPC 104E in that their L chains are nonglycosylated. Correcting for this difference a 9.8% carbohydrate content can be calculated for IgM. The hexose content of several mouse myeloma IgA proteins has been determined as equivalent to the values for human IgA (Underdown et al., 1971; Jaffe et al., 1971), for which a carbohydrate content of 8.0% can be calculated from the data of Baenziger and Kornfeld (1974). The carbohydrate content of IgG from various species including rodents is between 2.0 and 3.0% (Niedermeyer et al., 1971). Thus, the peak densities of the Ig preparation are in the order predicted from their carbohydrate contents.

For a homogeneous protein, the width of the distribution in a gradient is a function of the square root of the molecular weight (Meselson et al., 1957). The width of a protein distribution may be greater than expected because of a heterogeneity of density such as may arise from a variation in sugar content. Protein aggregation during centrifugation sharpens the peak. The relative importance of these three factors, molecular weight, heterogeneity, and aggregation, in determining the shape of the density distribution has not been determined.

Effect of Partial Reduction on Density. The degree of polymerization of Ig may affect the amount of hydration and ion binding and, as a result, the apparent density. Since we were

TABLE I: Density Shifts from β-Galactosidase and Densities of Immunoglobulins.

	Density Shift[a]		Density[b]	
	Control	NP40	Control	NP40
A.				
[^{125}I]IgG	0.0040	0.0050	1.320	1.321
[^{125}I]TEPC 15 IgA	0.0110	0.0095	1.327	1.325
[^{3}H]IgM	0.0135	0.0120	1.330	1.328
[^{125}I]MOPC 104E IgM	0.0160	0.0145	1.332	1.331
B.				
[^{3}H]IgM, extracellular	0.0140	0.0130	1.330	1.329
[^{3}H]IgM, extracellular, red. and alk.	0.0120	0.0115	1.328	1.328
[^{3}H]IgM, 14 min, intracellular		0.0030		1.319
[^{3}H]IgM, 4 h, intracellular		0.0080		1.324
C.				
[^{125}I]IgM, membrane		0.0050		1.321
[^{125}I]IgD, membrane		0.0100		1.326
[^{3}H]IgM, extracellular, red. and alk.		0.0120		1.328

[a] From β-galactosidase peak; g/cm^3; all values $\pm$ 0.0011 g/cm^3. [b] g/cm^3; all values $\pm$ 0.005 g/cm^3.

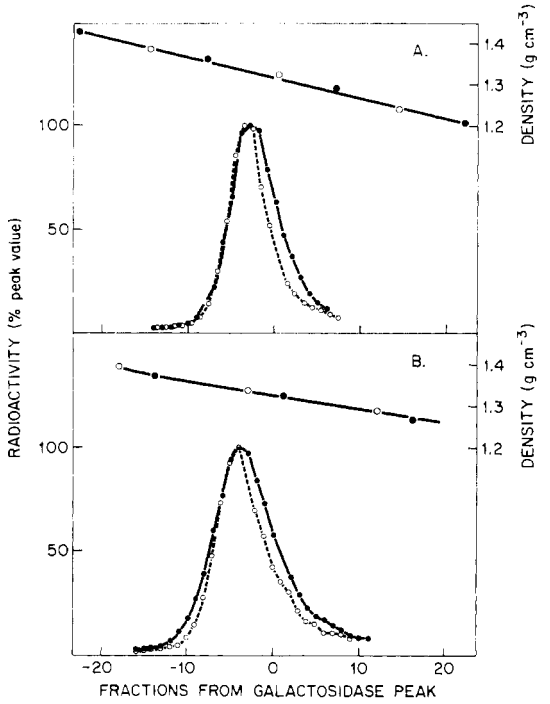

FIGURE 3: Density gradient centrifugation of ^{3}H-labeled secretions of mouse spleen cells. A partially reduced and alkylated ^{3}H-labeled secretion (solid circles, solid line) and an unreduced ^{3}H-labeled secretion (open circles, dashed line) were centrifuged in separate tubes with β-galactosidase. The difference between rabbit anti-κ and rabbit anti-$\phi\chi$ (control) precipitations (normalized to a peak value of 100%) is plotted. Other details are as described in Figure 1: (A) in the absence of detergent; (B) in the presence of NP40.

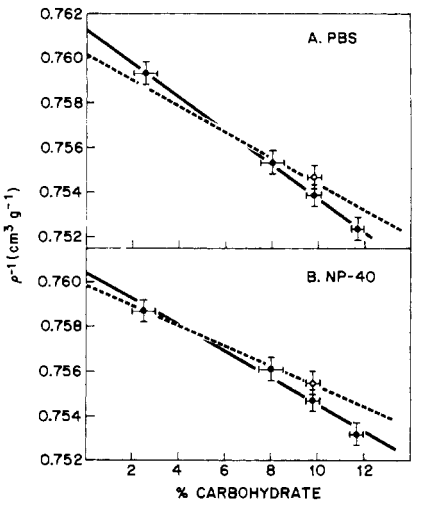

FIGURE 4: Relation between reciprocal density and carbohydrate content: (A) in the absence of detergent; (B) in the presence of NP40. Solid lines are least-square fits to the four points from Figures 1 and 2 (solid circles). Dashed lines are least-square fits using ^{125}I-labeled mouse γ-globulin, ^{125}I-labeled myeloma IgA, and partially reduced [^{3}H]IgM (open circles).

interested in the density of monomeric membrane IgM, we partially reduced a secretion containing [^{3}H]IgM by the procedure of Askonas and Parkhouse (1971). The partial reduction yielded μ_2L_2 and some higher polymers as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Melcher and Uhr, 1976). Partially reduced secretions were centrifuged in CsCl with β-galactosidase as a reference in the presence or absence of detergent. The profiles obtained are compared in Figure 3 with those of [^{3}H]IgM which had not been reduced. In this experiment the effect of NP40 on the peak density was not significant. The peak position of partially reduced IgM was slightly more dense than that of the unreduced molecule. The difference between Ig and β-galactosidase was 0.0012 g/cm^3 in the absence of NP40 and 0.0015 g/cm^3 in its presence (Table I, part B).

Relation between Density and Carbohydrate Content. The observed density, ρ_{obsd}, is related to the weight fraction of carbohydrate, χ, by the equation:

$$\frac{}{\rho_{obsd}} = \left(\frac{1}{\rho_1} - \frac{1}{\rho_2}\right)\chi + \frac{1}{\rho_2} \qquad (1)$$

where ρ_1 is the density of the oligosaccharide portion and ρ_2 is the density of the protein part of the glycoprotein. If the densities of the polypeptide portions of the different Ig's are the same, then a plot of $1/\rho_{obsd}$ against χ should yield a straight line. Such a plot is shown in Figure 4A using the peak densities from Figures 2 and 3B (detergent present). The vertical error bars reflect the error in density measurement. The horizontal error bars reflect the uncertainty in the assignment of values for the carbohydrate content. The solid lines are drawn by the method of least squares using the data for unreduced molecules. The dashed lines are drawn using data for IgA, IgG, and partially reduced [^{3}H]IgM. Within the error of the method, the experimental points fall on these lines, indicating that the major cause of density differences among these immunoglobulins is carbohydrate. The apparent buoyant density of the oligosaccharide was calculated from the slope and intercept of the solid line. The value of 1.45 g/cm^3 is less than the value for polysaccharides (1.6 g/cm^3) probably due to the firm binding of about 2 molecules of water per saccharide residue. We conclude that the polypeptide portions of Ig's are not significantly different in buoyant density from one another.

Intracellular IgM. If the above conclusions are correct, it should be possible to determine the approximate sugar content of an Ig by determining its density relative to β-galactosidase. We chose to test the method by determining the sugar content of intracellular [^{3}H]IgM. During biosynthesis of IgM in murine cells the terminal sugars fucose, galactose, and sialic acid are added close to the time of secretion (Melchers and Andersson, 1973; Melchers, 1973) so that the major intracellular intermediate lacks its complement of these sugars. Thus, the intracellular IgM of MOPC 104E myeloma cells has only 65% of the sugars that the secreted IgM molecule has (Melchers, 1973). If this is also true of normal mouse lymphoid cells, the intracellular IgM from these cells should be substantially less dense than the secreted IgM.

Accordingly, lysates of cells incubated for 4 h with [^{3}H]-tyrosine were centrifuged to equilibrium in CsCl. For comparison purposes lysates of cells incubated for only 14 min with radioactive precursor were also centrifuged. At short labeling times, the newly synthesized polypeptide chains should bear only a few oligosaccharide residues and have a lighter density. Fractions were assayed for [^{3}H]Ig by immunoprecipitation. Figure 5 shows the profiles obtained. The 14-min lysate had a broad distribution of Ig radioactivity. That of the 4-h lysate was narrower and was displaced in average position to the more dense side of the 14-min profile. The 14-min profile rises less steeply than the 4-h profile probably because there is little assembly of the newly synthesized H and L chains into H_2L_2 at the early time (as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis; data not shown).

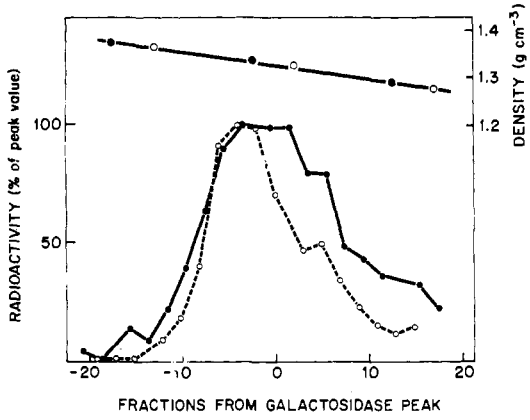

FIGURE 5: Density gradient centrifugation of lysates of spleen cells labeled for 14 min (solid circles, solid line) and 4 h (open circles and dashed line) with [^{3}H]tyrosine. The difference between rabbit anti-κ and rabbit anti-$\phi\chi$ (control) precipitations (normalized to a peak value of 100%) is plotted.

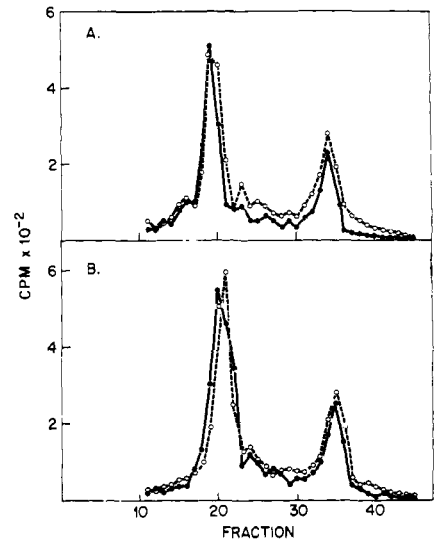

FIGURE 6: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 5% acrylamide of completely reduced Ig from cell lysates and cell supernatants after 4-h labeling with radioactive amino acids. Gels were run 2.5 h at 15 mA/gel: (A) ^{3}H-labeled lysate (dashed line) and ^{14}C-labeled secretion (solid line); (B) ^{14}C-labeled lysate (dashed line) and ^{3}H-labeled secretion (solid line).

In Table I, part B, the differences in density between the average peak position of the intracellular [^{3}H]IgM's and β-galactosidase are shown. The values for secreted IgM and partially reduced secreted IgM are included for comparison. Not only is the steady-state labeled intracellular IgM more dense than 14-min labeled IgM, but it is also significantly less dense than the secreted IgM. The observed density of 4-h lysate [^{3}H]Ig corresponds, according to Figure 4, to about 6% carbohydrate content, or three-fifths of the mature level. This agrees excellently with the value of 65% of the extracellular carbohydrate content which can be calculated from the data of Melchers (1973) on the carbohydrate composition of intra- and extracellular MOPC 104E IgM.

The conclusion that intracellular IgM from normal lymphoid cells is, on the average, underglycosylated is further substantiated by gel electrophoretic comparisons of heavy chains from secreted IgM and intracellular IgM. The mobility of Ig H chains in gels of 5% acrylamide monomer is sensitive to variations in carbohydrate content with the more highly glycosylated molecules having the lower mobility (Melcher and Uhr, 1976). Consequently, spleen cells were incubated for 4 h with [^{3}H]leucine and the intracellular IgM was recovered by immunoprecipitation. Another aliquot of cells was incubated with [^{14}C]leucine for 4 h and the extracellular IgM was recovered from the cell supernatant by immunoprecipitation. The precipitates were reduced and alkylated and run on the same 5% gel. Figure 6A shows the distribution of ^{3}H and ^{14}C radioactivity. In Figure 6B, the labels were reversed during the incubation to ensure that the results were not caused by an artifact of the isotope used. In both cases, ^{3}H and ^{14}C light chain radioactivity coincided. The lysate H chain, however, moved faster than the extracellular H chain. This can be interpreted to mean that extracellular H chain, and thus IgM, has substantially more carbohydrate than intracellular IgM, a result consistent with the density gradient result. Thus, an estimate of the carbohydrate content of an unknown Ig can be obtained by density gradient centrifugation. Since Ig can be precipitated with antisera from a complex mixture, small amounts of radioactive Ig can be assayed in the presence of other molecules, both radioactive and nonradioactive.

Trace Iodination. Having established that isopycnic centrifugation can detect density differences despite a heterogeneity in polypeptide structure, we can now turn our attention to membrane Ig's. Since the radioactive label used to locate membrane Ig and other Ig's is a heavy atom (125 amu) its effect on buoyant density must be considered. For a molecule the size of monomeric Ig's one iodine atom causes an increase of 0.0009 g/cm^3. In the above studies with trace iodnated Ig's the increase is less than 0.0005 g/cm^3 because of the state of polymerization of these Ig's as judged from peak widths and was thus ignored in the computations. In the case of membrane Ig, we calculate (from the specific activity of ^{125}I, the number of cells in a reaction, and the amount of radioactivity in membrane Ig in a typical reaction) that 1×10^3 atoms of iodine have been added to the Ig of each spleen cell. Estimates of the number of Ig molecules per cell are in the range of 1×10^5 (Warner, 1974). Thus, membrane Ig must have been trace iodinated, about 0.01 atom of iodine per molecule of Ig, so that the preponderance of radioactive membrane Ig molecules bears only one iodine atom.

Membrane Dissociation. In order to study the hydrodynamic properties of membrane Ig, it must first be dissociated from other membrane components. The extent of dissociation was examined in two ways. Cells were labeled with ^{125}I under conditions that resulted in some labeling of lipid. Identical aliquots were lysed in different concentrations of detergent. Two sets of immunoprecipitates were prepared from each lysate. One set was dissolved in 8 M urea and then diluted at least tenfold with 10% Cl_3CCOOH. Counts in the resulting precipitate were regarded as protein associated. The other set was extracted twice with chloroform–methanol (3:1) and the radioactivity in the organic phase was taken as a measure of the presence of membrane lipid in the immunoprecipitate. The amount of radioactive protein immunoprecipitated, although high at the lowest detergent concentration used, declined to a constant level at 0.5% NP40 (Figure 7). Membrane lipids are associated with the immunoprecipitates at low detergent concentrations. A concentration of 1.0% NP40 was necessary to bring this radioactivity down to a stable background level. Thus, a concentration of at least 1.0% is necessary to dissociate Ig from other membrane components when lysis is performed at 5×10^7 cells/ml.

A similar conclusion can be reached from density gradient

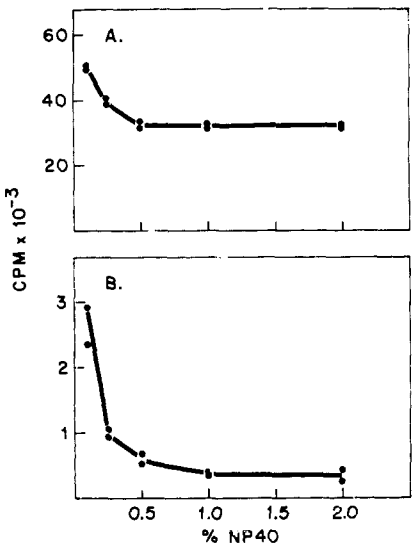

FIGURE 7: Effect of detergent concentration during lysis on protein and lipid in immunoprecipitates: (A) protein (Cl_3CCOOH precipitable radioactivity); (B) lipid (chloroform-methanol extractable radioactivity).

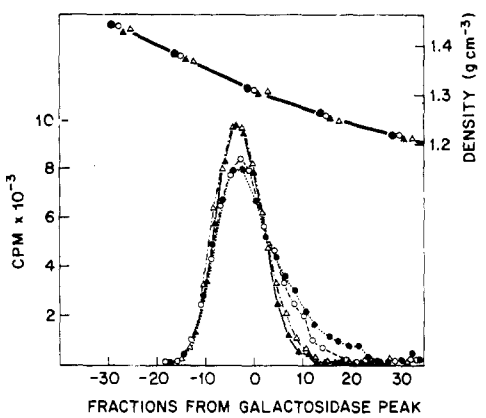

FIGURE 8: Effect of detergent concentration during lysis on buoyant density distribution of membrane Ig. Radioiodinated spleen cells were lysed at 0.1% (closed circles, dotted line), 0.5% (open circles, dashed line), 1.0% (open triangles, dashed line), and 2.0% (solid triangles, solid line) NP40. The difference between rabbit anti-κ and rabbit anti-$\phi\chi$ precipitations of three-drop fractions is plotted.

analysis. A population of iodinated spleen cells was divided into four aliquots which were lysed with different concentrations of NP40. The lysates were centrifuged to equilibrium in CsCl and the distribution of [^{125}I]Ig was determined by immunoprecipitation. A peak of radioactive membrane Ig was in each case observed in the same position (Figure 8). When cells were lysed at 0.1% NP40 considerable membrane Ig was present at lighter densities. The radioactivity in this region was reduced when lysis occurred at 0.5% NP40. This light radioactivity was further reduced by 1.0% NP40. The 2.0% curve is identical with the 1.0% curve indicating that 1% NP40 was sufficient to produce complete solubilization of membrane Ig from 5×10^7 cells/ml. Similar conclusions have been reached by Hart (1975) using other methods.

Buoyant Densities of Membrane IgM and IgD. In the above experiment, no effort was made to distinguish between te two classes of membrane Ig found on mouse spleen cells, IgM and IgD. We wished to determine the average density of membrane

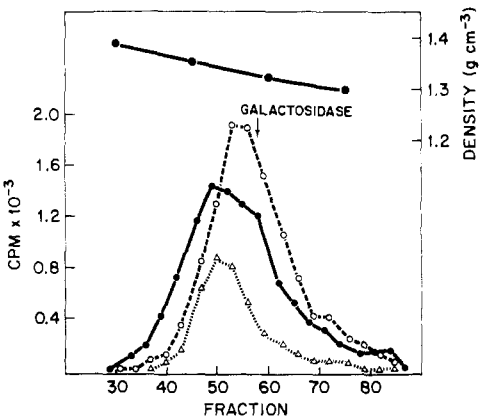

FIGURE 9: Density gradient centrifugation of an ^{125}I-labeled lysate and a partially reduced ^{3}H-labeled secretion. The differences between rabbit anti-μ and rabbit anti-$\phi\chi$ precipitations are plotted for ^{125}I-labeled membrane IgM (open circles, dashed line) and ^{3}H-labeled secreted IgM (open triangles, dotted line). The differences between rabbit anti-κ and rabbit anti-μ precipitations are plotted for ^{125}I-labeled membrane IgD (solid circles, dashed line). Two-drop fractions were used.

IgM and IgD separately so that they can be compared with the density of secreted IgM.

A lysate of iodinated cells was spun in CsCl together with β-galactosidase and a partially reduced secretion containing [^{3}H]IgM. Alternate fractions were precipitated with rabbit anti-μ, rabbit anti-κ, and rabbit anti-$\phi\chi$. The difference between the curve for rabbit anti-μ and rabbit anti-$\phi\chi$ was taken as IgM, while the difference between rabbit anti-κ and rabbit anti-μ precipitations was assumed to reflect IgD. In Figure 9, the density distributions of membrane IgD, membrane IgM, and secreted IgM are shown as is the peak position of β-galactosidase. The peak of membrane IgM was fairly symmetrical and on the average 0.005 g/cm^3 more dense than β-galactosidase (Table I, part C). The partially reduced secreted IgM in this experiment was 0.012 g/cm^3 more dense than β-galactosidase. The average density of membrane IgD was 0.010 g/cm^3 more dense than β-galactosidase and 0.005 g/cm^3 more dense than membrane IgM. The banding of membrane IgM and IgD was repeated three times with similar results. In two of these repeats the asymmetry of the IgD peak was reduced relative to that shown in Figure 9. The apparent heterogeneity of IgD may be due to a variation in carbohydrate content and/or in detergent binding.

Discussion

The simplest interpretation of the light density of membrane IgM (0.007 g/cm^3 lighter than partially reduced secreted IgM) is that detergent remains firmly bound to this IgM. The possibility that membrane IgM is severely underglycosylated (only about 3% sugar) is ruled out by two observations. Membrane μ chains behave on sodium dodecyl sulfate-polyacrylamide gel electrophoresis in different acrylamide concentrations very similar to secreted μ chains. H chains with low sugar content should move faster in gels of low acrylamide concentration (Figure 6). Fucose is a terminal sugar, one which is added late during the biosynthesis of secreted IgM (Melchers, 1973). The incorporation of fucose into membrane IgM has been reported (Vitetta and Uhr, 1974) indicating that at least some of the chains may be completely glycosylated. The possibility that membrane IgM is firmly bound to another protein which is unglycosylated and of fairly large mass is inconsistent with the peak width of membrane IgM. Such a complex should have

a higher molecular weight and thus a sharper peak. However, if either the Ig or the hypothetical binding protein were heterogeneous with respect to density, the inconsistency could be overcome. The possibility that membrane lipid still remains firmly attached to the Ig is ruled out by the observation that at the detergent concentrations employed, practically all lipid radioactivity has been removed (Figure 7). That some lipid is covalently attached to Ig has, however, not been excluded.

If our interpretation is correct and detergent is bound to membrane Ig, eq 1, where ρ_1 is now the density of the detergent (1.06 g/cm^3) and ρ_2 is that of IgM (1.328 g/cm^3, Table I), can be used to calculate the weight fraction of detergent in the complex. The value of ρ_{obsd} was taken as 1.320 g/cm^3 after correction of the value in Table I for the contribution of iodine. We calculate 2.5×10^{-2} g of detergent bound/g of IgM, or about 7 molecules of NP40 per IgM monomer. Although the calculation is subject to considerable error, both from the determined density values of IgM and since ρ_1 probably has a different value when bound to IgM than as a liquid, this level of detergent binding is nonetheless much less than that detected with several integral proteins as studied by gel filtration by Helenius and Simons (1975). The low level of binding may be due to two factors. One, our measurements were done in high concentrations of CsCl. Salt is known to affect the micellar properties of detergents (Helenius and Simons, 1975). Two, Ig is probably not a "deeply" buried membrane protein. Comparisons of the iodinatability of Ig on the surface of cells with Ig in solution indicate that most of the membrane Ig structure is exposed to solvent (Vitetta et al., 1971). Further, the observation of Fu and Kunkel (1974) of an antigenic activity of the C-terminal fragment of human μ chains which is not active in membrane bound IgM suggests that it is this C-terminal region only which is membrane active.

Although we and others have identified the two major classes of membrane Ig on murine cells as IgM and IgD, the evidence for the identification of IgD is not yet complete. Human IgD is known to have a higher percentage carbohydrate content than does human IgM (Spiegelberg, 1972). Our observation that mouse membrane IgD has a higher density than mouse membrane IgM indicates that IgD has a higher percentage content of carbohydrate or that membrane IgD binds much less detergent.

That the difference in carbohydrate content between intracellular and extracellular IgM as determined by buoyant density agrees with the value obtained by direct chemical analysis (Melchers, 1973) demonstrates the validity of using buoyant density to compare Ig's. Melchers (1973) has speculated that the addition of the remaining fucose and galactose residues may be a signal for polymerization of 7S IgM to 19S IgM and for secretion of IgM. Membrane IgM is monomeric, but seems by criteria of gel electrophoresis to have a complete complement of sugars. Thus, a complete complement of sugars is not a sufficient prerequisite for polymerization or secretion. The availability of J chain and a disulfide rearranging enzyme are necessary for polymerization (Della Corte and Parkhouse, 1973). It is unlikely that the final glycosylation reactions play a direct role in membrane attachment of Ig since these involve the sugars galactose and fucose which at least in the human IgM are part of the first three oligosaccharide chains from the N-terminal end (Putnam et al., 1973). The last two, and those most likely to be involved since Ig binding to membranes is probably via the C-terminal segment, are simple chains consisting of only mannose and *N*-acetylglucosamine residues. No large difference in these sugars was found by Melchers (1973).

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Reversible Inhibition of (Na^+,K^+)ATPase by Mg^{2+}, Adenosine Triphosphate, and K^+†

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ABSTRACT: Adenosine triphosphate (ATP) hydrolysis catalyzed by the plasma membrane (Na^+,K^+)ATPase isolated from several sources was inhibited by Mg^{2+}, provided that K^+ and ATP were also present. Phosphorylation of the adenosine triphosphatase (ATPase) by ATP and by inorganic phosphate was also inhibited, as was *p*-nitrophenyl phosphatase activity. (Ethylenedinitrilo)tetraacetic acid (EDTA) and catecholamines protected from and reversed the inhibition of ATP hydrolysis by Mg^{2+}, K^+, and ATP. EDTA was protected by chelation of Mg^{2+} but catecholamines acted by some other mechanism. The specificities of various nucleotides as inhibitors (in conjunction with Mg^{2+} and K^+) and as substrates for the (Na^+,K^+)ATPase were strikingly different. ATP, ADP, β,γ-CH_2-ATP and α,β-CH_2-ADP were active as inhibitors, whereas inosine, cytidine, uridine, and guanosine triphosphates (ITP, CTP, UTP, and GTP) and adenosine monophosphate (AMP) were not. On the other hand, ATP and CTP were substrates and β,γ-NH-ATP was a competitive inhibitor of ATP hydrolysis, but not an inhibitor in conjunction with Mg^{2+} and K^+. The Ca^{2+}-ATPase from sarcoplasmic reticulum and F_1, the Mg^{2+}-ATPase from the inner mitochondrial membrane, were also inhibited by Mg^{2+}. Catecholamines reversed inhibition of the Ca^{2+}-ATPase, but not that of F_1.

It has been recognized for many years that, although Mg^{2+} is required for activity of the (Na^+,K^+)ATPase,[1] elevated concentrations of this ion inhibit the enzyme activity (Wheeler and Whittam, 1962; Dunham and Glynn, 1961; Bond and Hudgins, 1975). The mechanism and the physiological significance of this inhibition have received little attention. Mg^{2+} has also been observed to inhibit F_1, the Mg^{2+}-ATPase of the inner mitochondrial membrane (Moyle and Mitchell, 1975). There has been no attempt to compare the mechanisms by which Mg^{2+} inhibits these different ATPases. The experiments presented here address these questions and are an outgrowth of our continuing efforts to study the roles of ATPases in active transport (Knowles and Racker, 1975; Racker and Fisher, 1975; Kagawa et al., 1973; Racker, 1972), and to study the regulation of ATPases in normal and cancer cells (Scholnick et al., 1973; Suolinna et al., 1974, 1975).

We have examined the mechanism of Mg^{2+} inhibition of the (Na^+,K^+)ATPase and found that inhibition required not only Mg^{2+}, but also ATP and K^+. In comparing several different ATPases, we have found distinct differences, as well as similarities, in the mechanisms by which Mg^{2+} inhibits. The concentrations of Mg^{2+} and ATP required for inhibition of the (Na^+,K^+)ATPase were well within their physiological ranges. This admits the possibility that these ligands may be regulators of the Na^+,K^+ transport system, of which the (Na^+,K^+)ATPase is a component.

Materials and Methods

Materials

$^{32}P_i$ was obtained from ICN, Irvine, Calif. [γ-^{32}P]ATP was prepared as described by Nelson et al. (1972). Deoxycholate was obtained from Schwarz/Mann, Orangeburg, N.Y., and recrystallized as described previously (Schneider et al., 1972).

Enzyme Preparations

Lamb Kidney (Na^+,K^+)ATPase. A kidney microsomal fraction was prepared by a modification of the procedure of Grisham and Barnett (1972), and stored at −70 °C. After thawing ten kidneys for 1 h in 0.32 M sucrose, 1 mM EDTA (pH 7.0) at 4 °C, the outer medulla was dissected with scissors and homogenized in a Waring blender at 4 °C with 9 ml of 0.32 M sucrose, 1 mM EDTA (pH 7.0) per gram of tissue. The homogenate was centrifuged for 15 min at 10 500 rpm in a No. 30 Spinco rotor. The pellet was discarded and the supernatant was filtered through five layers of cheesecloth and centrifuged for 60 min at 15 000 rpm in a No. 30 Spinco rotor. The resulting pellet was resuspended in 0.32 M sucrose, 10 mM

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[1] Abbreviations used are: ATPase, adenosine triphosphatase; EDTA, (ethylenedinitrilo)tetraacetic acid; NADH, reduced nicotinamide adenine dinucleotide; P_i, inorganic phosphate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; ATP, CTP, UTP, and ITP, adenosine, cytidine, uridine, and inosine triphosphates; AMP and ADP, adenosine mono- and diphosphates.